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Variation in fertilisation abilities between hemiclonal hybrid and sexual parental males of sympatric water frogs (*Rana lessonae*, *R. esculenta*, *R. ridibunda*)

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Abstract: In many species, males and females mate with multiple partners, which gives rise to sperm competition and multiple paternity. The experiments on water frogs presented here demonstrate that such sperm competition can affect the structure and dynamics of mixed species communities. The hybrid frog *Rana esculenta* (LR) mates with one of its parental species, usually *Rana lessonae* (LL) although in some areas *R. ridibunda* (RR), to regain the premeiotically eliminated parental genome ("hybridogenesis"). Mixed LL/LR-populations are stable although hybrid numbers should continuously increase at the expense of parental animals, because of differences in female fecundity and other factors. This would finally lead to the extinction of the sexual host, followed by that of the sexual parasite, unless the reproductive superiority of *R. esculenta* is reduced by other factors, such as lower hybrid male fertility. Eggs from LL- and LR-females were fertilised *in vitro* by single- and multi-male sperm suspensions of LL-, LR- and RR-males. In all experiments, the proportion of offspring sired by *R. esculenta* sperm was significantly lower than that sired by *R. lessonae* or *R. ridibunda* sperm. Gonad mass, sperm morphology, sperm swimming velocity, and sperm survival did not explain these differences in fertilisation success, nor did gamete recognition and compatibility. Sperm density was the only trait that paralleled fertilisation success; but it offers no explanation either, because densities were equalised for the *in vitro* fertilisations. In natural LL/LR populations, the significantly smaller amount, poorer competitive ability and lower long-term survival of *R. esculenta* compared to *R. lessonae* sperm will reduce the initial reproductive superiority of hybrids and contribute to the stabilisation of mixed water frog populations. Differences in fertilisation ability are also likely to be relevant for the structure and dynamics of several other systems with encounters between eggs and sperm from different genotypes, ecotypes, ploidy levels and/or species.

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Variation in fertilisation abilities between hemiclonal hybrid and sexual parental males of sympatric water frogs (*Rana lessonae*, *R. esculenta*, *R. ridibunda*)

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Abstract In many species, males and females mate with multiple partners, which gives rise to sperm competition and multiple paternity. The experiments on water frogs presented here demonstrate that such sperm competition can affect the structure and dynamics of mixed-species communities. The hybrid frog *Rana esculenta* (LR) mates with one of its parental species, usually *R. lessonae* (LL), although in some areas *R. ridibunda* (RR), to regain the premeiotically eliminated parental genome (“hybridogenesis”). Mixed LL/LR-populations are stable although hybrid numbers should continuously increase at the expense of parental animals, because of differences in female fecundity and other factors. This would finally lead to the extinction of the sexual host, followed by that of the sexual parasite, unless the reproductive superiority of *R. esculenta* is reduced by other factors, such as lower hybrid male fertility. Eggs from LL- and LR-females were fertilised in vitro by single- and multi-male sperm suspensions of LL-, LR- and RR-males. In all experiments, the proportion of offspring sired by *R. esculenta* sperm was significantly lower than that sired by *R. lessonae* or *R. ridibunda* sperm. Gonad mass, sperm morphology, sperm swimming velocity, and sperm survival did not explain these differences in fertilisation success; nor did gamete recognition and compatibility. Sperm density was the only trait that paralleled fertilisation success, but it offers no explanation either, because densities were equalised for the in-vitro fertilisations. In natural LL/LR

populations, the significantly smaller amount, poorer competitive ability and lower long-term survival of *R. esculenta* compared to *R. lessonae* sperm will reduce the initial reproductive superiority of hybrids and contribute to the stabilisation of mixed water-frog populations. Differences in fertilisation ability are also likely to be relevant for the structure and dynamics of several other systems with encounters between eggs and sperm from different genotypes, ecotypes, ploidy levels and/or species.

Keywords Sperm competition · Sexual parasite · Hybridogenesis · Populations dynamics · Community structure

Introduction

According to traditional sexual selection theory, a male's reproductive success increases with the number of females he mates with, whereas female success is related to the quality of the chosen male and/or his resources (reviewed by Andersson 1994). This concept is increasingly being extended to include the causes and consequences of male discrimination among females and female-female competition over access to several males (e.g. Cunningham and Birkhead 1998; Arnqvist and Nilsson 2000; Jennions and Petrie 2000; Bonduriansky 2001). Whichever is the choosing or the competing sex, the resulting conflict sets the stage for multiple mating and paternity. Reasons for such multiple mating can vary greatly as a consequence of the diversity of mating patterns, and they range from deliberate choice of multiple partners by females (e.g. Zeh and Zeh 2001) to sexual coercion by males (Thornhill 1980; Clutton-Brock and Parker 1995; Qvarnström and Forsgren 1998). The latter is particularly frequent where the operational sex ratio (OSR) is strongly male-biased and/or ecological conditions lead to clumped aggregations where female movements are restricted and males cannot effectively

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control mating access to females (Smuts and Smuts 1993; Clutton-Brock and Parker 1995; Gowaty 1997).

Lek-breeding anurans provide a typical example (Wells 1977). In frog choruses, often several satellite males gather around an amplexing pair or even cling to the same female, which results in “mating balls”. Because of this proximity, the released eggs are exposed to sperm from more than one male (sometimes belonging to more than one species), which can result in sperm competition and multiple paternity (Pyburn 1970; Coe 1974; Kasuya et al. 1987; Fukuyama 1991; Jennions and Passmore 1992, 1993; D’Orgeix and Turner 1995; Roberts et al. 1999). Laurila and Seppä (1998) suggested a high concentration of free-swimming sperm within a restricted mating area as another possible explanation for multiple paternity.

The likelihood of multiple paternity depends on several factors, including the frequency of multiple matings and differences among males in the amount and quality of sperm. The extent of multiple matings can be expected to vary with the distribution of matings in space and time (Emlen and Oring 1977). Among anurans, male-male competition, leading to mating balls and satellite males, is typically higher for “prolonged breeders” with their highly skewed OSR than for “explosive breeders” in which the short and highly synchronous mating activity leads to a more balanced OSR (Wells 1977). Male-specific differences in the amount and quality of sperm (Sivinsky 1984) can lead to varying fertilisation success among competing males.

Sperm competition theory (Parker 1984, 1990a, 1990b, 1993) assumes that sperm compete numerically, in a situation analogous to a raffle, in that the probability of fertilisation is proportional to the number of sperm ejaculated by each male. Because of limited resources, however, the production of many sperm may come at the expense of smaller sperm which, in turn, is related to reduced swimming velocity. Longer sperm are likely to generate greater flagellar forces (Katz and Drobnis 1990) and swim faster (Gomendio and Roldan 1991), both of which may be advantageous if sperm compete actively or “race” to fertilise. Several studies, ranging from *Caenorhabditis elegans* (LaMunyon and Ward 1998) through *Drosophila* (Joly et al. 1991), butterflies (Gage 1994) and sea urchins (Levitan 2000) to mammals (Gomendio and Roldan 1991), have indeed shown that fertilisation success increases with sperm size and/or speed and that differences in size are largely a result of differences in tail length, the most variable part of sperm (Cummins and Woodall 1985).

Studying the relative success of competitors is usually done within the framework of its fitness consequences for the individual (behavioural ecology) or the spreading of alleles within and between populations of the same species (population genetics). It can, however, also be relevant for understanding the structure and dynamics of mixed-species communities (ecology). This is particularly true for cases in which at least one sex (usually males) mates fairly indiscriminately (as in anurans) and/or in

species with external fertilisation where eggs can be reached by both homo- and heterospecific sperm. Depending on the relative frequencies of within- and between-species fertilisations, development of the respective parental—and possibly arising hybrid—populations may take totally different routes. An excellent model system for studying the implications of differential fertilisation for the structure and dynamics of mixed-species populations are the European water frogs used in this investigation.

The water-frog complex

Rana esculenta (genotype *LR*) is an interspecific hybrid, originally produced through matings between *R. lessonae* (genotype *LL*) and *R. ridibunda* (genotype *RR*). During gametogenesis, the hybrid excludes one of its parental genomes premeiotically, duplicates the remaining one and transmits it clonally to eggs and sperm. This special reproductive mode, known as “hybridogenesis” (Schultz 1969; Tunner 1974), requires that *R. esculenta* lives in mixed populations with the parental species whose genome it eliminates. Such mixed populations have been described for many areas of Europe. Although the geographical distribution of genome exclusion is complicated, there is a tendency for hybrids in eastern Europe to eliminate the *R* genome and live and mate with *R. ridibunda* (*R-E*-system), and in western Europe to eliminate the *L* genome and live and mate with *R. lessonae* (*L-E*-system) (for reviews, see Günther 1990 for example). Because mixed *L-E*-populations are the most widespread ones and typical for study areas in Switzerland, we focus on this system.

In such mixed *L-E*-populations, heterotypic matings between *R. esculenta*, as a sexual parasite, and *R. lessonae*, as a sexual host, produce viable hybrid offspring (Table 1). Homotypic matings between *LL* males and females lead to *LL* offspring. Homotypic *LR* pairings produce *RR* tadpoles, but these usually die during the larval stage or shortly after metamorphosis, probably because of homozygosity of deleterious mutations that have accumulated on the clonally transmitted *R* genome (Berger 1977; Graf and Müller 1979; Semlitsch and Reyer 1992; Vorburger 2001; Guex et al. 2002).

According to Table 1, only one of the possible four mating combinations produces *LL* offspring, whereas two pairings produce *LR* offspring. This numerical superiority of the hybrid, plus the fact that *R. esculenta* females produce larger clutches than *R. lessonae* (Berger and Uzzell 1980; Reyer et al. 1999), and *R. esculenta* larvae

Table 1 Possible mating combinations and resulting offspring types in mixed water-frog populations; † indicates that *R. ridibunda* tadpoles from this mating combination are not viable

Females/males	<i>R. lessonae</i> (<i>LL</i>)	<i>R. esculenta</i> (<i>LR</i>)
<i>R. lessonae</i> (<i>LL</i>)	<i>R. lessonae</i> (<i>LL</i>)	<i>R. esculenta</i> (<i>LR</i>)
<i>R. esculenta</i> (<i>LR</i>)	<i>R. esculenta</i> (<i>LR</i>)	<i>R. ridibunda</i> (<i>RR</i>) †

perform better than *R. lessonae* larvae under most ecological conditions (Semlitsch and Reyer 1992; Semlitsch 1993; Semlitsch et al. 1997), gives the hybrid a reproductive advantage. If mating were random, this would initially increase the hybrid's relative abundance in mixed populations, but in the long run it would dilute the parental species out of the population and, in the absence of the necessary sexual host, lead the hybrid to extinction too. This, however, is not what we observe in nature. Several studies have documented that the composition of mixed populations varies among ponds, but is remarkably stable over time within ponds (Berger 1977; Blankenhorn 1977; Holenweg Peter et al. 2002). According to theoretical models (Hellriegel and Reyer 2000; Som et al. 2000) and empirical studies, mate choice is one of the most important factors that enhances the production of parental relative to hybrid offspring, and hence reduces the numerical surplus of hybrids expected under random mating. In twofold choice experiments, both *R. esculenta* and *R. lessonae* females showed a preference for *R. lessonae* males (Abt and Reyer 1993; Roesli and Reyer 2000; Engeler and Reyer 2001). Although, under more natural conditions, competition between the indiscriminately mating males affects the relative frequency of the various pairing combinations (Bergen et al. 1997), females can choose copulation partners, to some extent, by approaching calling *R. lessonae* rather than *R. esculenta* males (Roesli and Reyer 2000) and/or avoiding contact with the hybrid males by fleeing from their advances (Abt and Reyer 1993). If this does not prevent amplexus, they can provoke fights between males leading to the displacement of the amplexing male (H.-U. Reyer, unpublished data) or exert "cryptic" choice, i.e. withhold eggs when amplexed by hybrid males (Reyer et al. 1999).

In this study, we suggest and investigate an additional mechanism that might affect the relative frequencies of hybrid and parental offspring: differential fertilisation success. If matings involving *R. esculenta* males and/or females lead to a lower proportion of fertilised eggs than homotypic *LL* matings, this will result in a further reduction in the production of hybrids and contribute to the stabilisation of mixed *LE*-populations. Within this general hypothesis, we addressed the following specific questions: (1) Are there differences in fertilisation rates in single- and multiple-species sperm mixtures? (2) If yes, are there correlates of these differences in the amount, size and/or velocity of sperm?

Because previous studies had shown higher variance in fertility of *R. esculenta* males relative to *R. esculenta*

females (e.g. Berger 1973; Günther 1973), suggesting abnormalities in male but not female gametes, we also included sperm from *R. ridibunda* males to test for potential fertilisation differences between clonal *R* sperm from the hybrid and recombined *R* sperm from the parental species.

Methods

Animals

All frogs used in the experiment were captured during the breeding season in May 2000. *R. lessonae* (*LL*) and *R. esculenta* (*LR*) were obtained from a pond near Hellberg (47°10'N, 8°49'E) in the Canton of Zürich, Switzerland, which has an estimated frog population of 700–800 adults with an *LR/LL*-ratio of 3:1 (H.-U. Reyer, unpublished data). *R. ridibunda* (*RR*) were collected from a pond near Yverdon (46°43'N, 6°34'E) in the Canton of Waadt, Switzerland. The water-frog population there is large (>2,000 adults) and consists of *R. ridibunda* only (Grossenbacher 1988; Vorburger 2002). Between the capture procedure and the start of the experiment, frogs were kept in boxes (40×37×60 cm) at 10°C, separated by sex and species.

In-vitro fertilisation

Experimental design

To test for fertilisation abilities and sperm competition, we carried out three different in-vitro experiments (Table 2). Eggs from one female were fertilised: (a) with sperm from one male in the single-male experiments, (b) with the sperm from two males, mixed in a ratio of 50:50%, in the two-male experiment, and (c) with a 33:33:33% sperm mix from three males in three-male experiments. Each of the three experiments was replicated seven times. Within replicates, eggs from each female were subjected to all seven sperm suspensions that result from the three treatments (columns in Table 2), and sperm from each male was used to fertilise the eggs of each female (rows in Table 2). This "half-sib design", which was chosen to control for differences in fertilisation probability of eggs and fertilisation ability of sperm among individuals, resulted in 14 crossing combinations with 42 in-vitro fertilisations each in experiments a and b (2 female types*3 suspensions*7 replicates), and 14 in experiment c (2 female types*1 suspension*7 replicates).

Fertilisation procedure

Although crosses can be achieved by letting water frogs mate naturally in outdoor or laboratory containers, this approach was not suitable for our project. It neither would have allowed us to standardise sperm densities across sperm suspensions, nor would it have produced equal proportions of spermatozoa from different males within suspensions. Therefore, we carried out in-vitro fertilisation, following the procedure described by Berger et al. (1994). Two days before the experiment, females and males were

Table 2 Experimental design for the in-vitro fertilisation of eggs from parental (*LL*) and hybrid (*LR*) females with sperm suspensions of one male type (1–3), and mixtures of two males (4–6) and three

males (7), respectively (*LL* *R. lessonae*, *LR* *R. esculenta*, *RR* *R. ridibunda*). Subscripts $_n$ refer to the number of replicates, with $_n$ running from 1 to 7

Females	One male			Two males			Three males
	1	2	3	4	5	6	7
<i>LL_n</i>	<i>LL_n</i>	<i>LR_n</i>	<i>RR_n</i>	<i>LL_n+LR_n</i>	<i>LL_n+RR_n</i>	<i>LR_n+RR_n</i>	<i>LL_n+LR_n+RR_n</i>
<i>LR_n</i>	<i>LL_n</i>	<i>LR_n</i>	<i>RR_n</i>	<i>LL_n+LR_n</i>	<i>LL_n+RR_n</i>	<i>LR_n+RR_n</i>	<i>LL_n+LR_n+RR_n</i>

weighed, and their snout-vent lengths (SVL) were measured to the nearest 0.1 mm. Both sexes were subcutaneously injected with approximately 100 μ l/10 g bodyweight of the fish hormone LHRH (H-7525, Bachem Bioscience) in a concentration of 1 mg/100 ml isotonic saline solution. In females, this induces ovulation within 48 h; in males, it has a positive effect on sperm motility. Thereafter, animals were kept individually in covered plastic containers (20×11.5×7.5 cm) fitted with a moist paper towel.

Two days later, males were killed in a plastic container filled with 3-aminobenzoic acid ethyl ester (MS222, 500 mg/100 ml). Both testes were dissected, weighed to the nearest milligram and temporarily stored in 13 ml Holtfreter's solution, before they were crushed, thoroughly carved up with pincers and the sperm released into a petri dish with 1 ml Holtfreter's solution. For each male, we determined the density of mobile sperm per 1 ml by counting the moving spermatozoa in 1 μ l of the resulting suspension in a Neubauer chamber. For the single-male experiments (a), this 1 ml represented the stock suspension. Stock suspensions for the multi-male experiments (b, c) were created by mixing sperm from two and three males, respectively. For calculating the required quantities of *LL*, *LR* and *RR* sperm, sperm numbers of the male with the lowest density (e.g. 52×10^4 /ml in *LR*) were doubled to give the total amount of sperm available for a crossing (104×10^4 /ml). In the two-male experiment (b), 1 ml sperm suspension of the male with the lowest sperm density constituted 50% of the calculated total amount; the amount of sperm suspension representing the 50% of the other male (e.g. an *LL* with 56×10^4 /ml) was calculated through a simple rule of 3 ($x = 1 \times 52 \times 10^4 / 56 \times 10^4 = 0.93$ ml). In the three-male experiments (c), the total number of sperm was calculated in the same way as in the two-male experiment, but each frog contributed only one-third to the total. In all three experiments, the stock suspensions were then increased to a total volume of 10 ml by adding tap water that had been exposed to air for about 24 h.

Each of the resulting seven sperm suspensions (Table 2) was placed into two petri dishes, one for *LL* eggs and one for *LR* eggs. Into each petri dish, we stripped a portion of eggs (between 100 and 200) from 1 *LL* and 1 *LR* female, respectively. The stripping was done by gently widening the female's cloaca with wet, blunt forceps while slightly pressing the female's body. To control for potential effects of sperm age on fertilisation ability, the sequence of adding eggs to the suspensions was shifted from suspension 1–7 in replicate 1 to 7–1 in replicate 7. The whole procedure and the subsequent maintenance of eggs and larvae was done at room temperature (21°C). After an in-vitro fertilisation series had been completed, females were put back into large boxes filled with water, kept in the cold room until all females had been used, and then returned to their pond of origin.

Fertilisation success is evident after 20–50 min when the black animal hemisphere of fertilised eggs rotates to the top. At this time, we added enough aged water to cover the eggs completely. To calculate fertilisation success, we first counted the total number of eggs in the petri dish and then, after the first cleavage was visible (usually after 3 h), all fertilised and unfertilised eggs. The next day, the eggs were transferred from petri dishes into plastic containers (20×11.5×7.5 cm) that were filled with aged water to a height of 2 cm. Water in the containers was changed periodically during development of the eggs. After hatching, the tadpoles were fed every day with powdered fish food (Sera Micron) and raised for 30–37 days.

Paternity determination

In order to determine fertilisation success of *LL*, *LR* and *RR* males in the single-, two- and three-male experiments, we analysed the genotypes of parents and offspring from tissue samples. From adult females we took a toe, from previously killed males a foot, from large tadpoles a piece of the tail fin, and from small ones the whole body. For this purpose, 20 offspring from each cross of the single- and 2-male experiment and 30 offspring from the 3-male experiment, were randomly caught and killed with MS222. All tissue samples from adults and larvae were put individually into

Eppendorf tubes, which were then stored at -80°C . Samples were analysed for genetic variation using (1) cellulose-acetate electrophoresis or (2) DNA analysis.

1. Cellulose-acetate electrophoresis—following the technique described in detail by Hebert and Beaton (1993), we tested all samples for the following six enzymes (abbreviations and Enzyme Commission number shown in parentheses): Aspartate Amino Transferase (AAT; EC no. 2.6.1.1), Glucose-6-Phosphate-Isomerase (GPI; EC no. 5.3.1.9), Lactate Dehydrogenase (LDH; EC no. 1.1.1.27), Mannose-6-Phosphate-Isomerase (MPI; EC no. 5.3.1.8), Phosphoglucosmutase (PGM; EC no. 5.4.2.2) and 6-Phosphogluconate Dehydrogenase (6PGDH; EC no. 1.1.1.44). The alleles shown by these enzymes were known from earlier studies of genetic variation in our source populations (Hotz 1983; Vorburger 2001). Enzyme profiles were defined as vs (very slow), s (slow), f (fast) and vf (very fast). If one enzyme, or a combination of enzymes, showed sufficient genetic variation among adults (e.g. *LL* eggs=vs, *LL* sperm=f, *LR* sperm=s and *RR* sperm=vf), we used the profiles from the cellulose acetate electrophoresis for paternity determination. If this was not the case, we had to determine paternity by DNA analysis.
2. DNA analysis—DNA was extracted from all samples using the QIAamp DNA mini kit (Qiagen). DNA concentration was determined via spectrophotometry, and polymerase chain reaction (PCR) amplification was done using primers and protocols of Garner et al. (2000), Zeisset et al. (2000) and Hotz et al. (1997). Electrophoresis was done using the SEA 2000 advanced submerged gel electrophoresis apparatus (Elchrom Scientific, Switzerland). PCR products were run on EL300 or EL600 gels (Elchrom Scientific, Switzerland), depending on the expected allele sizes. Electrophoresis was done at 100 V for 90–120 min, again depending on expected allele size and also depending on the gel type used. After electrophoresis, gels were stained for 45 min using SYBR gold nucleic acid stain (Molecular Probes) and then destained with water for 30–60 min. Gels were imaged on a transilluminator using a digital video camera, and alleles were scored against the M3 marker (Elchrom Scientific, Switzerland) by eye. Initially, all of the adults from every cross were amplified using all the available primer pairs. From these PCR primers, loci diagnostic for each cross were then used to amplify DNA from the offspring of the same cross. Offspring profiles were then compared to the profiles of the potential parents, and paternity was determined via band-sharing.

Testis and sperm characteristics

In order to investigate the reasons for potential differences in fertilisation rates, we compared the three male types with respect to (a) the amount of sperm, (b) sperm morphology, (c) sperm velocity and (d) sperm survival. For logistic reasons, preparation of sperm suspensions for fertilisation and investigation of sperm velocity and survival could not be done with the same males. Therefore, new frogs were caught to obtain these data. Males were collected from the same populations as those used for the other investigations, and were injected with LHRH, killed with MS222 and dissected as described above for in-vitro fertilisation.

Amount of sperm

For each of the 21 males (7 per species) used for the in-vitro fertilisation, we measured testis mass to the nearest milligram and determined the density of mobile sperm as described above. In addition, testis size and sperm density were also measured for the 12 frogs that were dissected for the sperm-survival measurements. This resulted in testis size and sperm-density data for 33 males (11 each for *LL*, *LR* and *RR*).

Sperm morphology

Two to three drops of the single-male sperm suspensions 1–3 used for the in-vitro fertilisation were pipetted onto microscope slides and allowed to dry for later sperm-size measurements. Five slides from each of the seven males per species were examined under a Wild M3C microscope fitted with a Donpisha 3CCD camera that relayed images to a PC running Optimas 6.5 software (MediaBio-cybernetics, USA), which was used to make all measurements. For each frog, we measured the flagellum and head length (μm) of 100 sperm and calculated the total sperm length and the tail-to-head length ratio. Unfortunately, sperm of one *LL*, three *LR* and one *RR* could not be measured because the drying process had torn the sperm apart. This left us with sample sizes of 6, 4 and 6, respectively.

Sperm velocity

Data on sperm velocity were collected on five *LL*, eight *LR* and nine *RR*. For measuring sperm speed, we prepared sperm solutions as described above and let the sperm swim in “tunnels”, prepared by adhering two 0.5-cm-wide parafilm strips to the surface of a glass slide and then melting a cover glass onto the two strips. The sperm suspension was pipetted underneath the cover glass taking advantage of cohesion forces. The slide was placed under a microscope fitted with a TV camera connected to a videotape recorder. The videotapes were digitised with NIH Image software (National Institutes of Health, Bethesda, USA), which resulted in stacks of 120 frames per 60 s. By following individual sperm cells and recording their locations for 8–10 min (i.e. 960–1,200 frames/sperm), we obtained a distance and a speed measure for each sperm. For each frog, we measured, on average, the speed of 26 sperm (range 12–59) and then used mean speed per frog in the statistical analyses.

Sperm survival

For measuring sperm survival, sperm suspensions were prepared by crushing the dissected testes into petri dishes with 0.5 ml aged tap water. To filter out larger tissue pieces that might hinder subsequent sperm counting, we washed the sperm suspension through a filter (hole diameter: 100 μm) into an Eppendorf tube with aged tap water and diluted it to 0.5 ml. At this point, time started running for the survival measurements. Sperm suspension (5 ml) was pipetted from Eppendorf tubes onto microscope slides after 5 min (t_0), and again after 3, 8, 24, 48 and 96 h; 5 ml of 1% neutral red solution was pipetted onto each sperm solution. This vital stain is taken up by living cells only (Romeis 1948) and, thus, allowed us to reliably distinguish between living and dead spermatozoa at a 800-fold magnification using a Zeiss light microscope. For each male (four *LL*, four *LR* and four *RR*), we prepared four slides and counted spermatozoa in ten randomly chosen vision-fields per slide.

Statistical analysis

For the in-vitro fertilisation experiment, we performed an analysis of variance (ANOVA) to examine the proportion of fertilised eggs [arcsin(sqrt)-transformed] in relation to female type (*LL*, *LR*), female individual within types (=replicates), and the seven sperm suspensions shown in Table 2, which reflect different combinations of male types. We also used uni- and multivariate analyses of (co)variance to investigate the effects of male type (*LL*, *LR*, *RR*) and male size on testis size and sperm characteristics. Because data for density of mobile sperm were obtained in 2 different ways, namely through counting (a) mobile sperm for the in-vitro fertilisation ($n=21$) and (b) stained sperm at time t_0 for survival measurements ($n=12$), we standardised data within each data set separately (mean=0, SD=1) and then used the standardised sperm densities of all 33 data sets as the dependent variable in the

ANOVA. Pairwise comparisons were done through Scheffe's tests. All statistical analyses were performed with program SYSTAT 8.0 for Windows (1998).

Results

In-vitro fertilisation

The average proportion of fertilised eggs, pooled over all experiments, was 81% for *R. esculenta* and 83% for *R. lessonae* females. There were no differences in mean fertilisation success between the two female types ($P=0.493$), but differences among females within types were significant ($P<0.001$; Table 3; Fig. 1a). Fertilisation was also significantly affected by the type of the sperm suspension ($P<0.001$; Fig. 1b). *LR* sperm in the single-male experiment (suspension 2) fertilised a lower proportion of eggs than the other suspensions (all pairwise $P<0.003$, Scheffe's test), whereas these other suspensions did not differ in their fertilisation success (all pairwise $P\geq 0.682$). The interaction between sperm suspension and female type was not significant ($P=0.127$), which indicates that *LL*- and *LR*-eggs did not differ in their response to pure and mixed sperm suspensions.

The mean fertilisation success of *LR* (60.0%) relative to that of *LL* (82.9%) and *RR* (82.0%) was used to calculate the expected success of *LR* in mixed sperm suspensions. The resulting values are 42% for both 2-male combinations *LL/LR* [$=60/(60+82.9)$] and *RR/LR* [$=60/(60+82.0)$] and 27% for the 3-male experiment [$=60/$

Table 3 Results from four analyses of variance. The ANOVA for fertilisation rate in (a) relates the proportion of fertilised eggs [arcsin(sqrt)-transformed] to female type (*LL*, *LR*), individual female within female type, sperm suspension (1–7, see Table 2) and the interaction between sperm suspension and female type. The ANOVAs for testis size and sperm density in (b) and (c) relate these two measures of sperm amount (testis size and sperm density) to male type with testis size as a covariable in the latter ANOVA (c). The fourth MANOVA in (d) relates sperm morphology to male type (*LL*, *LR*, *RR*). The usual statistics for the univariate analyses (sums-of-square, *df*, *F* and *P*) are given; the multivariate test statistic (Wilks' lambda) is also shown for sperm morphology

	Sums-of-squares	df	F	P
(a) Fertilisation rate, $R^2=0.781$				
Female type	0.018	1	0.475	0.493
Female individual (type)	7.172	12	16.340	<0.001
Suspension	2.053	6	9.422	<0.001
Suspension×Female type	0.388	6	1.728	0.127
(b) Testis size, $R^2=0.467$				
Male type	14.490	2	13.155	<0.001
(c) Sperm density, $R^2=0.567$				
Male type	7.866	2	8.503	0.001
Testis size	11.433	1	24.717	<0.001
(d) Sperm morphology				
Sperm length	0.000	2	8.336	0.005
Tail/head ratio	0.101	2	1.174	0.340
Wilks' lambda	0.323	4, 24	4.556	0.007

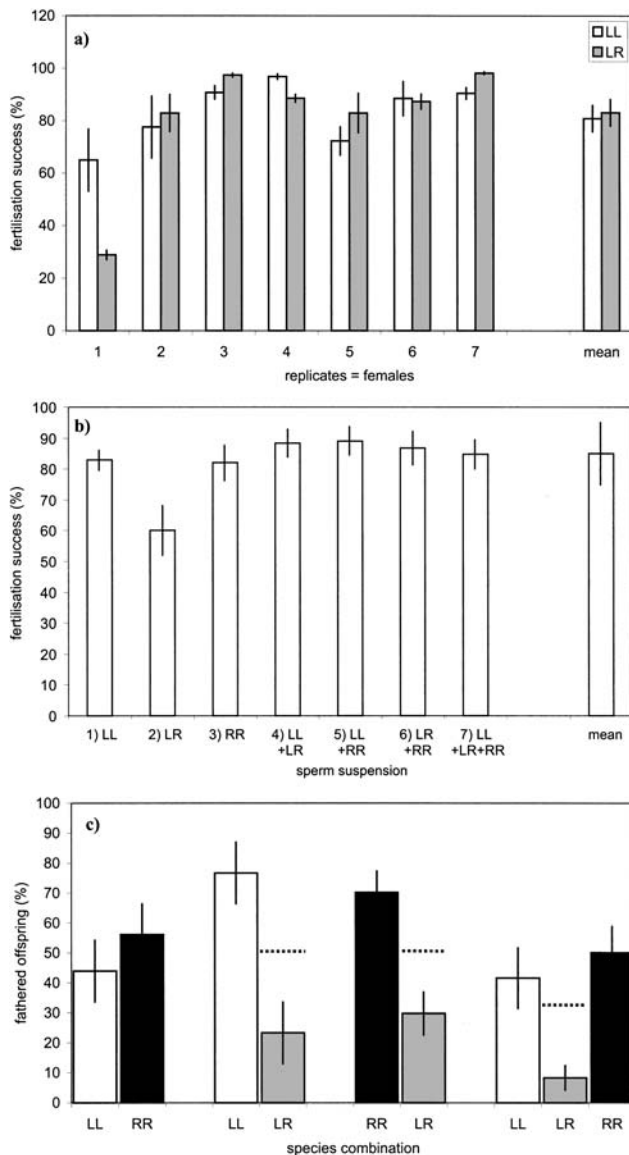


Fig. 1a–c Percentage of in-vitro fertilised eggs in relation to female type and individual female (a), type of sperm suspension (b) and species combination in the mixed sperm suspensions 4–7 (c). Shown are least-square means \pm SE from the ANOVA in Table 3. In c the dotted lines indicate the proportions expected for *R. esculenta* on the basis of its fertilisation success in the single-species experiments with suspensions 1–3; for calculation of these expectations, see Results (LL *Rana lessonae*, LR *R. esculenta*, RR *R. ridibunda*)

(60+82.9+82.0)]. In both experiments, the actual percentage of offspring fathered by *R. esculenta* was not only lower than the percentage fathered by *R. lessonae* and *R. ridibunda*; it was also significantly lower than expected (all $P < 0.05$, paired t -tests; Fig. 1c). Thus, in competition with sperm from the parental species, the hybrid sperm was even less successful than when alone. LL and RR sperm were equally competitive. Their success in the two- and three-males experiments did not differ from expectations based on the one-male experiment (Fig. 1c).

Testes and sperm characteristics

In our search for potential correlates of the hybrid males' lower fertilisation success, we compared (a) amount of sperm, (b) sperm morphology and (c) sperm speed and survival among the three male types. Results from the respective (M)ANOVA are shown in Table 3.

Amount of sperm

This was measured by testis size and sperm density. Testis size increased from LL through LR to RR (Fig. 2a left; Table 3; $P \leq 0.027$ for both pairwise comparisons involving LL and $P = 0.095$ for comparison LR vs RR). This increase apparently reflects the parallel increase in body size and the fact that testis size correlates positively with body size ($r = 0.927$, $n = 33$, $P < 0.001$). When testis mass is expressed as a percentage of body mass, the effect of male type on testis size disappears ($F_{2,43} = 1.674$, $P = 0.199$).

Density of mobile sperm increased with testis size, but was also related to male type (Table 3). Relative to testis size, sperm density decreases from LL through RR to LR (bars in Fig. 2a right). Pairwise comparisons reveal that sperm densities in *R. lessonae* are significantly higher than in *R. esculenta* ($P = 0.001$) and tend to be higher than in *R. ridibunda* ($P = 0.105$), whereas the last two species do not differ ($P = 0.352$). In absolute terms (i.e. without correction for testis size), sperm density decreased in the order $RR > LL > LR$ (line in Fig. 2a right) with a significant difference between RR and LR ($P = 0.037$) and no difference for the two comparisons involving LL (both $P \geq 0.335$).

Thus, the sperm-density patterns (Fig. 2a right) parallel the fertilisation success (Fig. 1b, c) in that the hybrid *R. esculenta* performs worse than the two equally successful parental species. Yet, the differences in fertilisation success cannot be explained through differences in sperm densities because these had been standardised prior to in-vitro fertilisation and differed neither among suspensions ($F_{5,42} = 0.291$, $P = 0.915$) nor between single- and multi-male experiments ($F_{1,42} = 0.748$, $P = 0.392$; ANOVA, multiple $R^2 = 0.103$).

Sperm morphology

Sperm morphology, measured by the total sperm length and the ratio between the sperm tail and head length, also differed among male types ($P = 0.007$; Table 3). According to the univariate analyses, this difference resulted from a difference in total sperm length ($P = 0.005$); the tail/head ratio was similar in all three species. Total sperm length was significantly lower in LL than in LR and RR (both $P < 0.04$), but did not differ between the last two species ($P = 0.863$; Scheffe's pairwise tests) (Fig. 2b left). Again, the difference seems to mainly reflect a difference in body size, because the significant male type effect on sperm

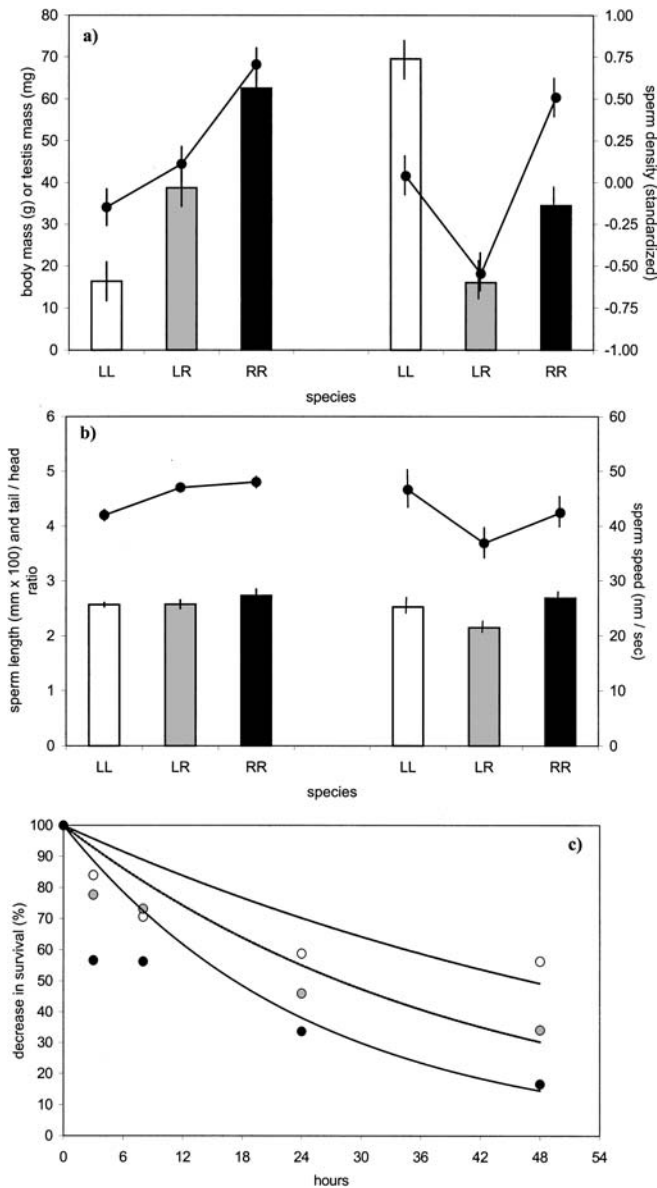


Fig. 2a–c Measures for amount of sperm (a), sperm morphology and speed (b) and sperm survival (c) for male *Rana lessonae* (LL), *R. esculenta* (LR) and *R. ridibunda* (RR); a and b show least-square means \pm SE from the analyses of variance in Table 3 for the following variables: a left: line body mass, bars testis mass; right: line absolute sperm density, bars sperm density corrected for testis size; b left: line total length of sperm, bars length ratio between sperm tail and head; right: line maximum sperm speed, bars mean sperm speed. Some SE in b are so small that they disappear behind the symbols. In c, exponential functions were fitted to the decrease in viable sperm of *R. lessonae* (white symbols and dotted line), *R. esculenta* (grey symbols and broken line) and *R. ridibunda* (black symbols and solid line), respectively

length disappears ($P=0.157$) when SVL is entered as a covariable into the MANOVA.

Sperm velocity

Sperm velocity seemed to be lower in the hybrid *R. esculenta* than in the two parental species (Fig. 2b, right), but statistical analyses revealed no significant male effect for either mean or maximum sperm speed ($F_{2,19}=1.054$, $P=0.368$ and $F_{2,19}=0.710$, $P=0.504$, respectively; ANOVA).

Sperm survival

Figure 2c shows the decrease in sperm survival over time, expressed as the percentage of vital sperm at 3, 8, 24 and ≥ 48 h, relative to the percentage that was vital immediately after preparing the suspension ($t_0=100\%$). Sperm survival declines exponentially for all three male types, but at different rates. Longevity is highest in *R. lessonae*, lowest in *R. ridibunda* and intermediate in the hybrid *R. esculenta*.

Discussion

Fertilisation success

The in-vitro fertilisation experiment revealed neither a difference in the fertilisability of LL and LR eggs nor in the fertilisation success of the *R. lessonae* and *R. ridibunda* males. Hybrid *R. esculenta* males, however, were consistently less successful than males of the two parental species. In the single-species experiment, hybrid sperm suspensions fertilised, on average, 27% fewer eggs (Fig. 1b), and in mixed sperm suspensions, allowing competition with sperm from one or both of the parental species, their success was even further decreased (Fig. 1c).

Previous investigations on differences in fertilisation success between hybrid and parental males are mostly anecdotal and yield no clear picture, except that fertility in male hybrids seems to be high. Observations range from total sterility in dissected hybrid males (Günther 1990) through a 50% fertilisation rate in some natural matings (H.-U. Reyer, unpublished data) to no obvious differences in artificial fertilisation experiments (Berger et al. 1994). In the only study that tested fertilisation abilities directly, Berger and Rybacki (1992, 1994) found that, in mixed sperm suspensions, the *L*-spermatozoa of *R. lessonae* fertilised more eggs than the *R*-spermatozoa of *R. ridibunda* and *R. esculenta*. They attributed the difference to a 16% higher DNA content in *R*- than in *L*-sperm, which makes the *R*-sperm heavier, slower and competitively inferior to the lighter and faster *L*-sperm (Berger and Rybacki 1994). Their study suffered from a number of technical and statistical shortcomings, however, including differences in the “freshness” of sperm

suspensions, an unbalanced experimental design, no statistical tests and no measurement of the supposedly important difference in swimming activity. Our results reveal no major difference in fertilisation ability between the lighter *L*- and the heavier *R*-sperm. There are differences, however, between clonal *R* from hybrids and both *L* and *R* from the parental species. This difference is paralleled by some of the sperm features discussed below.

Explanations for the observed variation in fertilisation success

With the exception of tail/head ratio and velocity of sperm, the three male types differ significantly in a number of characteristics, including the amount of sperm (measured through testis size and sperm density), sperm length and sperm survival (Fig. 2). None of these differences, however, can explain why the fertilisation success of hybrid sperm was lower than that of sperm from the two parental species.

The amount of sperm offers no explanation, because sperm densities in the in-vitro experiments were standardised and differed neither among suspensions nor between single- and multi-male experiments (see Methods). In situations without such experimental standardisation (e.g. in natural ponds), however, *R. esculenta* is likely to be at a disadvantage compared to *R. lessonae* and *R. ridibunda*. Densities of mobile sperm were lower in hybrid than in parental males, not only relative to testis size, but also in absolute terms, even though hybrid testis was intermediate (Fig. 2a).

Differences among male types in sperm morphology also do not parallel the observed fertilisation success. The ratio between sperm tail and sperm head length did not differ among species, and total sperm length, a potential correlate of speed (for references, see Introduction) was significantly lower in *R. lessonae* than in the other two species. *LL*-sperm was, nevertheless, equally successful as *RR*-sperm and even more (not less) successful than the larger *LR*-sperm. Given this lack of a relationship between fertilisation success and sperm morphology, it is not too surprising that we found no significant difference in sperm velocity, although larger sample sizes are needed to test the impression from Fig. 2b that maximum and mean sperm speed may be lower in *R. esculenta* than in the two parental species.

For sperm survival, we have no data for the initial period during which in-vitro fertilisations were performed. Three and more hours after preparing suspensions, however, survival of *LR*-sperm was intermediate between, not lower than, *LL*- and *RR*-sperm (Fig. 2c). We see no reason to assume a different pattern in the first few minutes to hours. Moreover, differences in survival rates are unlikely to explain the observed differences in fertilisation success because sperm from the two parental species fertilised equally well, despite their difference in survival. In natural *LL/LR*-systems, however, *R. esculenta*

might be at a disadvantage compared to *R. lessonae* if free-swimming sperm does gain fertilisation sensu Laurila and Seppä (1998).

Differences in sperm traits are only one potential mechanism that might explain differences in fertilisation rates. Another mechanism is sperm selection through females. Such “cryptic” female choice for good genes and/or genetic compatibility has been demonstrated in numerous species with internal fertilisation (reviewed by Eberhard 1996; Birkhead 1998; Tregenza and Wedell 2000), but is unlikely to operate when fertilisation is external, as in water frogs. A third mechanism that can work with external fertilisation is gamete recognition. An increasing number of studies, especially on marine organisms, reveal that successful fertilisation requires molecules in the sperm and egg to recognise each other in a taxon-specific manner (e.g. Vacquier et al. 1990; Palumbi 1994; Rakitin et al. 1999) and/or that eggs release substances that attract the appropriate sperm (Al-Anzi and Chandler 1998). Our study provides no direct evidence for such taxon-specific sperm recognition; the lack of a significant female type \times sperm suspension interaction (Table 3) even shows that *L*- and *R*-eggs were fertilised at equal rates by *L*- and *R*-sperm from the parental species. Nevertheless, we cannot rule out the possibility, that in *LL/LR*-systems, selection has resulted in eggs that specifically reduce the probability of being fertilised by sperm of those males that should be avoided. For both *R. lessonae* and *R. esculenta* females, these are the hybrid males.

Implications for natural populations

Whatever the mechanisms behind the observed fertilisation differences are, they can be expected to affect the structure and dynamics of natural populations. Higher fecundity and usually better larval performance in hybrid than in parental animals, plus the fact that in mixed *LL/LR* populations, two mating combinations produce *R. esculenta* whereas only one produces *R. lessonae* offspring (Table 1), would lead to ever-increasing hybrid proportions and finally drive both the sexual host *LL* and the sexual parasite *LR* to extinction. According to recent models (Hellriegel and Reyer 2000; Som et al. 2000), stability depends on a complex network of mating patterns, fecundity, larval performance and terrestrial dispersal, but requires that the initial reproductive superiority of the hybrid be reduced in one way or another. With respect to mating, previous studies had found such reduction through assortative mating and clutch size adjustment (Abt and Reyer 1993; Reyer et al. 1999; Roesli and Reyer 2000; Engeler and Reyer 2001). By demonstrating 27% lower fertilisation success of the existing sperm in single-male matings (Fig. 1b), even lower success in competitive multi-male situations (Fig. 1c), a smaller total amount of sperm (Fig. 2a right), and lower long-term survival of free-swimming sperm (Fig. 2c) relative to *R. lessonae*, this study has identified

additional mechanisms that potentially reduce hybrid success and, thus, contribute to stability.

We feel that the results and conclusions from our study are relevant not only for water frogs and the few other hybridogenetic species (Dawley and Bogart 1989; Bullini 1994; Alves et al. 2001), but also for all those cases where behavioural control of paternity is limited and, hence, probabilities of “random” encounters between eggs and sperm are high. Such cases range from plants exposed to a mixture of own, con- and heterospecific pollen (reviewed by Ellstrand 1992), through aquatic animals with external fertilisation of eggs surrounded by floating clouds of sperm from different populations, ploidy levels and/or species (e.g. Palumbi 1994; Taborsky 1998; Lambert 2000; Ritterbusch and Bohlen 2000; Garcia-Varquez et al. 2002) to species where fertilisation is internal, but females are forcefully copulated by several males (Thornhill 1980; Clutton-Brock and Parker 1995; Qvarström and Forsgren 1998), or mate with heterospecific males when conspecifics are rare (Bergen et al. 1997; Wirtz 1999; Ribi and Oertli 2000; Hettzey and Pearman 2003). In all these and similar cases, the relative fertilisation success of gametes from different genotypes, ecotypes and/or taxa will determine the number of in- and outbred individuals originally produced, including the number of inter-specific hybrids, which are abundant in plants, but also occur in substantial proportions in many animal taxa (reviewed by Arnold 1997). Together with selection acting upon them, this will affect the stability and dynamics of populations and even mixed-species communities. We feel, therefore, that the role of sperm competition in an ecological context requires more attention.

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